EXHIBIT 21
PART 3

substrate regions in the entire cell population which has reacted, and r represents the critical number of substrate sites which must react for lysis of a given cell. The term c_x^m is given by the expression,

$$\frac{m!}{x!\,(m-x)!}$$

It is assumed that the average extent of reaction, p, would be proportional to the amount of complement added. Alberty and Baldwin also showed that inhomogeneity of red cells can be incorporated in the theory by variation of r. From analyses of titration data describing the reaction of EA with C′, i.e., the over-all action of C′, Alberty and Baldwin concluded that r has a value of about 10.

The validity of this theory has been questioned on several grounds (23). The assumption that p is proportional to the amount of complement added represents an oversimplification since it has been shown, as pointed out in an earlier section, that the lytic effectiveness of complement is dependent upon its concentration. The validity of the multiple-hit or cumulative damage concept, on which the theory is based, became doubtful when it was observed that in the limited complement system the *absolute* number of cells lysed by a given concentration of complement is independent of the total number of sensitized cells in the reaction system. (This is true only if the percentage of lysis is low and if the sensitization of the cells is in the range from about 100 to 500 molecules of antibody per cell.) If one accepts a value of r = 10, it would be predicted from the Alberty-Baldwin theory that a quantity of complement capable of lysing 2.5 billion out of a total population of 5 billion cells (i.e., 50% hemolysis), would hemolyze only about 200 or 300 million cells when the total population of the sensitized cells is 10 billion (i.e., 2 or 3% lysis). Instead, about 25% lysis is produced when a total of 10 billion cells is used (cf. Table 4). This observation was the first suggestion that lysis by complement might be a one-hit reaction.

When the sequence of action of the complement components had become definitely established, and when methods for isolation of the intermediates became available, the way was opened for development of a rigorous theoretical treatment. Instead of trying to construct a theory for the entire hemolytic process, attempts were made to analyze the individual reaction steps. The first opportunity to do this presented itself following the discoveries of the intermediate product EAC′1, 4, 2 and of the E* sampling procedure. It was then thought, erroneously, that the conversion of EAC′1, 4, 2 to E* by C′3 constitutes a single reaction step; we were not aware at the time of the work of Da Costa Cruz and Penna (95) on the duality of C′3.

This error went undetected for several years because of an additional mistake, viz. the finding that formation of E* from EAC′1, 4, 2 by C′3 proceeds without lag (23). It has been pointed out already in an earlier section that this error arose from faulty sampling techniques in the kinetic analyses.

Eventually, it was discovered by Rapp (90), through detailed theoretical and kinetic analyses, that the action of C′3 on EAC′1, 4, 2 comprises more than one step. In turn, this led to isolation of C′3a and C′3b (90, 152). Consequently, the one-step theoretical model described in (23) is invalid.

Before proceeding with further discussion of the theoretical analysis of the C′3 reaction, in terms of a two-step model, it will be convenient to turn attention to the transformation of EAC′1, 4 to EAC′1, 4, 2 by C′2. The opportunity to study this simpler case presented itself when purified C′2 became available.

Theoretical Analysis of C′2 Reaction

On the basis of information presented in earlier sections, it is believed that the

following model describes the conversion of $SA_2C'1, 4$ to $SA_2C'1, 4, 2$:

$$\begin{array}{ccccc} (A) & & (B) & & (C) \\ SA_2C'1, 4 & + & C'2 & \xrightarrow{k_1} & SA_2C'1, 4, 2 \\ \uparrow & & & & \downarrow \\ & & & \xleftarrow{k_2} & \end{array}$$

Definitions: (The mathematical symbols given below should not be confused with the immunological notations in cases where the same letters are used.)

- A = average number of $SA_2C'1, 4$ per cell at time, t
- A_o = initial value of A
- B = number of $C'2$ molecules (expressed on a "per cell" basis) at time, t
- B_o = initial value of B
- C = average number of $SA_2C'1, 4, 2$ per cell at time, t
- k_1 = specific rate constant of formation of $SA_2C'1, 4, 2$
- k_2 = specific rate constant of decay of $SA_2C'1, 4, 2$

Let us assume that A remains constant at the initial value A_o; this condition can be approached quite closely since it is possible to run analyses with a large excess of $SA_2C'1, 4$ over $C'2$.

The rate equations will be:

$$-\frac{dB}{dt} = k_1A_oB$$

$$\frac{dC}{dt} = k_1A_oB - k_2C$$

On integration one obtains:

$$B = B_oe^{-k_1A_ot} \qquad [2]$$

$$C = \frac{k_1A_oB_o}{k_1A_o - k_2}\left[e^{-k_2t} - e^{-k_1A_ot}\right] \qquad [3]$$

Equation [3] is not applicable when $k_1A_o = k_2$. For this special case:

$$C = k_2B_ote^{-k_2t} \qquad [4]$$

Let $y_{1,4,2}$ represent the proportion of cells in the state $EAC'1, 4, 2$, and let us assume that one $SA_2C'1, 4, 2$ is necessary and sufficient to transform a cell to the state $EAC'1, 4, 2$ (which means that the cell is potentially lysable by $C'3$). It follows from the Poisson distribution that

$$y_{1,4,2} = 1 - e^{-C} \qquad [5]$$

or

$$-\ln(1 - y_{1,4,2}) = C \qquad [5']$$

Substituting [5'] in [3] and [4], respectively,

$$-\ln(1 - y_{1,4,2}) = \frac{k_1A_oB_o}{k_1A_o - k_2}\left[e^{-k_2t} - e^{-k_1A_ot}\right] \qquad [6]$$

$$-\ln(1 - y_{1,4,2}) = k_2B_ote^{-k_2t} \qquad [7]$$

For experimental evaluation, samples withdrawn from the reaction mixture are treated with $C'3$ in high concentration so that practically all $EAC'1, 4, 2$ will lyse. Under these conditions, the degree of lysis, y, will be approximately equal to $y_{1,4,2}$. Precise evaluation requires use of the theoretical equation for the action of $C'3$, which is given below.

The time, t_{max}, at which C reaches its maximal value, C_{max}, is given by the following relation:

$$t_{max} = \frac{\ln k_1A_o - \ln k_2}{k_1A_o - k_2} \qquad [8]$$

except when $k_1A_o = k_2$. Under these conditions, $t_{max} = \frac{1}{k_2}$.

Thus, t_{max} is independent of B_o, and this has been confirmed experimentally (cf. Fig. 59). Furthermore, if A_o is kept constant, it has been found that C_{max} varies linearly with B_o, as predicted by equation [3] (cf. Fig. 62). Thirdly, t_{max} decreases as A_o is increased, as indicated by equation [8] (cf. Fig. 60).

In titrations of $C'2$, B_o in equations [6] or [7] is the desired value. Measurements are made with incubation time equal to

t_{max}. For calculation, it is necessary to know the value of k_1A_o applicable to the EAC'1, 4 preparation in use. This may be derived from t_{max} by equation [8]. In addition, it is necessary to know k_2 at the temperature of experimentation. The value of this constant is obtained from decay experiments. As noted before, small variations in the value of k_2 have been observed, and therefore, it is preferable to measure it for each preparation of EAC'1, 4. The cause of these variations of k_2 is not known.

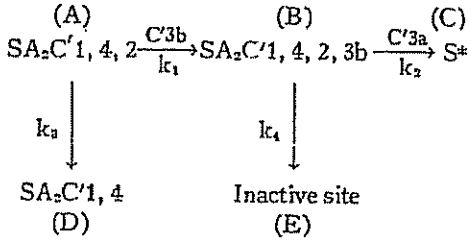
Estimates of C'2 obtained in this manner should be regarded as *minimal* values, since the possibility of unfruitful reactions resulting in destruction of C'2 cannot be excluded. For this reason, analytical results have been expressed in terms of "effective" C'2 concentration.

Theoretical Analysis of C'3 Reaction

According to Taylor and Leon (153), EAC'1, 4, 2 reacts with C'3a to form an intermediate EAC'1, 4, 2, 3a, which reacts with C'3b to form E*. On the other hand, Rapp's experiments indicate that C'3b reacts before C'3a. Attempts to isolate the intermediate product have met with much difficulty, and consequently, it has not yet been possible to resolve this matter definitively. For this reason, at present it is not possible to study the reactions of C'3a and C'3b as individual steps, and the scheme to be presented will deal with a two-step model, comprising the action of both C'3a and C'3b on EAC'1, 4, 2.

In the design of this two-step model by Rapp, the following assumptions are made: (1) decay steps proceed according to first-order kinetics; (2) interaction of sites with C' components is pseudo-first order under conditions where consumption of the latter is negligible; (3) formation of one S* on a given cell is a necessary and sufficient condition for conversion of that cell to E*. This assumption appears valid on the basis of the findings for the C'2 and C'4 reactions, which have been presented.

The model is represented schematically as follows:



Definitions:

A = average number of $SA_2C'1, 4, 2$ per cell at time, t

B = average number of $SA_2C'1, 4, 2, 3b$ per cell at time, t

C = average number of S* per cell at time, t

D = average number of $SA_2C'1, 4$ per cell at time, t

E = average number of decayed $SA_2C'1, 4, 2, 3b$ per cell at time, t

k_1 = specific reaction rate constant of $SA_2C'1, 4, 2, 3b$ formation

k'_1 = reaction rate constant for $SA_2C'1, 4, 2, 3b$ formation; $k'_1 = k_1[C'3b]v$

k_2 = specific reaction rate constant of S* formation

k'_2 = reaction rate constant of S* formation; $k'_2 = k_2[C'3a]v$

k_3 = specific rate constant of decay of $SA_2C'1, 4, 2$

k_4 = specific rate constant of decay of $SA_2C'1, 4, 2, 3b$

[C'3a] and [C'3b] are the concentrations of these factors per unit volume of serum or reagent and v is the volume used experimentally. The differential equations arising from this model are as follows:

$$-\frac{dA}{dt} = (k'_1 + k_3)\,A$$

$$\frac{dB}{dt} = k'_1\,A - (k'_2 + k_4)\,B$$

$$\frac{dC}{dt} = k'_2 B$$

At $t = 0$, $A = A_o$, $B = C = D = E = 0$ provided that $k'_1 + k_3 \neq k'_2 + k_4$, these differential equations have the following solutions:

$$A = A_o e^{-(k'_1 + k_3)t} \quad [9]$$

$$B = \frac{k'_1 A_o}{(k'_1 + k_3) - (k'_2 + k_4)} \left[e^{-(k'_2 + k_4)t} - e^{-(k'_1 + k_3)t} \right] \quad [10]$$

$$C = \frac{k'_1 k'_2 A_o}{(k'_1 + k_3)(k'_2 + k_4)} \left[\frac{(k'_1 + k_3)(1 - e^{-(k'_2 + k_4)t}) - (k'_2 + k_4)(1 - e^{-(k'_1 + k_3)t})}{(k'_1 + k_3) - (k'_2 + k_4)} \right] \quad [11]$$

For convenient experimental evaluation, let us consider end-point measurements, i.e., allow t to become sufficiently large so that an estimate of the limiting value of C (C_{max}) can be made, which is:

$$C_{max} = \frac{k'_1 k'_2 A_o}{(k'_1 + k_3)(k'_2 + k_4)} \quad [12]$$

Inversion and simplification yields a more convenient relation:

$$\frac{1}{C_{max}} = \frac{1}{A_o} + \frac{1}{A_o} \left[\frac{k_3}{k'_1} + \frac{k_4}{k'_2} + \frac{k_3 k_4}{k'_1 k'_2} \right] \quad [13]$$

Since $k'_1 = k_1 [C'3b] v$ and $k'_2 = k_2 [C'3a] v$, in experiments with a mixture containing both C'3a and C'3b, this equation may be written as follows (here, v represents the volume of the mixture):

$$\frac{1}{C_{max}} = \frac{1}{A_o} + \frac{1}{A_o} \left[\frac{k_3}{k_1 [C'3b] v} + \frac{k_4}{k_2 [C'3a] v} + \frac{k_3 k_4}{k_1 k_2 [C'3a] [C'3b] v^2} \right] \quad [14]$$

This equation may be expressed in linear form, as follows:

$$v \left(\frac{1}{C_{max}} - \frac{1}{A_o} \right) = \frac{1}{A_o} \left[\frac{k_3}{k_1 [C'3b]} + \frac{k_4}{k_2 [C'3a]} \right] + \frac{1}{A_o} \left[\frac{k_3 k_4}{k_1 k_2 [C'3a] [C'3b]} \right] \cdot \frac{1}{v} \quad [15]$$

For experimental evaluation of this relationship, C_{max} is derived from observed values of y, the proportion of cells lysed, by the Poisson distribution, on the assumption that one S* suffices for E* formation, and hence, lysis of a cell, i.e., $C_{max} = -\ln(1-y)$. Furthermore, it is necessary to determine the value of A_o which yields the best straight line when $v \left(\frac{1}{C_{max}} - \frac{1}{A_o} \right)$ is plotted on an arithmetic grid against $\frac{1}{v}$.

Since C'3a and C'3b have been separated from one another, it is also possible to study: (1) the case where C'3a is kept constant and the concentration of C'3b is allowed to vary, and (2) the case where C'3b is kept constant and the concentration of C'3a is allowed to vary. The following equations are applicable to these cases:

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$$\frac{1}{C_{max}} = \frac{1}{A_o}\left[1 + \frac{k_4}{k_2[C'3a]}\right] + \frac{k_3}{A_o k_1[C'3b]}\left[1 + \frac{k_4}{k_2[C'3a]}\right] \cdot \frac{1}{v_b} \quad [16]$$

and,

$$\frac{1}{C_{max}} = \frac{1}{A_o}\left[1 + \frac{k_3}{k_1[C'3b]}\right] + \frac{k_4}{A_o k_2[C'3a]}\left[1 + \frac{k_3}{k_1[C'3b]}\right] \cdot \frac{1}{v_a} \quad [17]$$

where, v_a and v_b are the volumes of the C'3a and C'3b fractions, respectively. For experimental evaluation, plot $\frac{1}{C_{max}}$ vs. $\frac{1}{v_b}$ or $\frac{1}{v_a}$ on an arithmetic grid.

By combining equations [5] and [12], we obtain:

$$y_{max} = 1 - e^{-\frac{k'_1 k'_3 A_o}{(k'_1 + k_3)(k'_3 + k_4)}} \quad [18$$

which is a sigmoidal function describing the relation between the proportion of cells lysed at the endpoint, y_{max}, and the concentrations of C'3a and C'3b. This is the long-sought explanation for the sigmoidal shape of complement titration curves.

From equation [12] it is possible to evaluate the proportion of $SA_2C'1, 4, 2$ converted to S* by any given concentration of the C'3 factors. It is also evident from [12] that this proportion is independent of A_o, and depends only on the concentration of C'3. This is the justification for the treatment of decay data which has been presented.

In evaluating equation [15] an estimate of A_o is obtained, which should agree with estimates of the number of $SA_2C'1, 4, 2$ by the decay method, or alternatively, by preparing EAC'1, 4, 2 from EAC'1, 4 with a known number of C'2 molecules. Precise experimental comparisons of this kind are not yet available, but approximate estimates have been found to agree.

Finally, it should be noted that these theoretical analyses have taken no account of the known variation of susceptibility of red cells to lysis by antibody and complement. The basis of this variation is not known, but it appears likely that it arises from unfruitful reactions. One need only assume that with more resistant cells the proportion of unproductive encounters is greater than with less resistant cells. Such a mechanism admits the possibility of multiple hits, but it does not constitute a cumulative process and in this sense the characteristics of the reaction would still be those of a one-hit process. The term "one-hit" then refers to a non-cumulative model, which means that successive hits constitute independent events. This formulation would recognize the possibility that some sites that have reacted with each of the complement components are not S*.

EXPERIMENTAL PROCEDURES FOR KINETIC ANALYSIS

Equipment

For kinetic analysis it is convenient to use Erlenmeyer flasks partially submerged in a constant temperature water bath from a short clamp attached to a horizontal rod which rotates back and forth through an angle of about 70 to 80° at a rate of about thirty to forty complete strokes per minute. The suspension arrangement is constructed so that the bottom of the Erlenmeyer flask does not emerge from the water at the end of the stroke and the water level of the bath should be brought as high as possible without danger of

shipping water. The agitation of the mechanical rocker should create a gentle swishing action of the contents of the flask adequate to maintain the cells in uniform suspension; violent shaking with turbulence should be avoided. The water bath can be constructed from a rectangular aquarium tank made of glass which will permit observation of the contents of the flasks during an experiment. It has been found convenient to install the water bath on a table equipped with casters so that the entire set-up can be wheeled into a cold room when necessary. For temperature regulation a mercury thermoregulator with relay connected to a low-lag immersion heater is suggested. A stirrer or circulating pump should be attached for continuous mixing of the contents of the water bath to maintain uniform temperature. For ease of setting pipettes to the mark on withdrawal of samples, it is helpful to install a shielded fluorescent lighting fixture slightly above and behind the bath. An electric switch should be installed on the table in front of the bath so that the mechanical shaker can be turned off and on conveniently each time a sample is withdrawn. Attachment of an electric timer reading in tenths and hundreds of a minute will also be a convenient feature.

For operation below room temperature insert a copper coil in the water bath and circulate cold water or refrigerant continuously at a rate so adjusted that the capacity of the intermittent electric immersion heater controlled by the thermoregulator will not be exceeded.

On an adjacent table set up a battery of six to eight table centrifuges. This will permit centrifugation of samples immediately upon withdrawal from the reaction mixtures.

General Procedure for Kinetic Measurements of the Reaction Between EA and C′

In a typical experiment, 10 ml. of erythrocyte suspension, standardized photometrically to contain 5 x 10^8 cells per ml., and 10.0 ml. of the desired hemolysin dilution, are pipetted into a thoroughly cleaned 125 ml. Erlenmeyer flask suspended from the mechanical rocker in the water bath. If the same hemolysin concentration is to be used in all reaction mixtures, it will be convenient to sensitize the cells prior to delivery into the reaction flasks. Close flasks with rubber stoppers. After about eight to ten minutes' shaking, 5.0 ml. of a suitable dilution of guinea pig serum, also pre-warmed to the desired temperature, are added as a source of complement, and timing of the reaction is begun when half of the complement dilution has been delivered. This method of choosing "zero-time" is, of course, somewhat arbitrary. In order to reduce the uncertainty of timing it is convenient to use "blow-out" pipettes which run out in about ten seconds; another few seconds should be allowed for drainage before the last drop is blown out of the pipette. In experiments in which accuracy of timing is critical, calibrated syringes with wide bore hypodermic needles should be used for delivery of the last reagent. In this way 1 or 2 ml. of reagent can be delivered within one second. As a guide with respect to quantitation of reagents it can be indicated that dilutions of guinea pig C′ ranging from 1/200 to 1/500 should be suitable for optimally sensitized sheep erythrocytes. For studies of the limited antibody region, a 1/15 or 1/30 dilution of guinea pig serum should be adequate. Prior to use, the guinea pig serum must be absorbed at least twice with washed sheep erythrocytes in order to remove most of the natural hemolysin. The reaction mixtures are shaken continuously during the entire experiment, except for brief interruptions during withdrawal of samples. At the chosen time intervals, samples of 0.5 ml., 1 ml., or 1.5 ml., depending on the purpose of the experiment, are withdrawn by means of an accurately calibrated pipette and immediately delivered into and mixed with several volumes of ice-cold

"stop-diluent," For this purpose a solution containing 0 12 *M* NaCl and 0.015 *M* sodium citrate, or a corresponding solution containing 0.005 *M* EDTA may be used. A rack of labelled tubes containing "stop-diluent" should be set up in advance in an ice-water bath, so that samples can be withdrawn in rapid succession, if necessary. The test tubes for receiving the samples should be sufficiently large so that the stop-diluent and sample can be mixed rapidly and efficiently by twirling immediately upon delivery from the pipette. The sampling pipettes should have delivery periods of about ten seconds from mark to tip, and the time recorded for each sample is usually taken at the moment when one-half of the contents of the pipette has run out. The pipette is allowed to drain for a few seconds after the fluid has reached the tip, and is then blown out, in accordance with the procedure used for its calibration. Efforts should be made to adhere to uniform technique of sampling in order to keep the volumetric and timing errors to a minimum. After each sample, the pipette should be rinsed once with 0.15 *M* NaCl solution, allowed to drain, and after blowing out the last drop the tip should be wiped with dry filter paper. If several different reaction mixtures are run simultaneously, it is usually advisable to use a separate sampling pipette for each flask. Under all circumstances a separate pipette must be used for "blank" reaction mixtures, i.e., cells alone, cells + antibody, or cells + complement.

In analyses in which complement is the limiting reagent, citrate or EDTA are ineffective for stoppage and, therefore, prompt termination of the reaction process depends solely on chilling and dilution. The highest degree of dilution which is experimentally practicable is about 1/21 (i.e., 0.5 ml. sample delivered into 10 ml. of stop-diluent in a 15 x 125 millimeter tube; a larger amount of fluid cannot be mixed quickly and effectively in this tube, and a larger tube cannot be used because of the limitation of the table centrifuges). It is necessary to centrifuge samples immediately upon withdrawal and admixture with stop-diluent, and this is possible only if a battery of table centrifuges is available. In experiments requiring withdrawal of samples in very rapid succession, e.g., at one-half minute intervals, it may be necessary to centrifuge samples in pairs; in this event, the first member of the pair should be held in an ice-water bath until the second tube is ready for the centrifuge. Brief delays not exceeding one minute are probably insignificant, provided the sample is kept ice-cold. In experiments involving complement as the limiting reagent, or in any other kind of kinetic experiment in which citrate or EDTA are ineffective for stoppage, it is essential that the table centrifuges are kept as cold as possible. It is advisable to run the experiment in a cold room for this reason. This is not necessary when hemolysin is the limiting reagent; under these conditions chelation is effective for stoppage.

After centrifugation, the supernatant fluid should be poured off promptly from each of the samples into a dry labeled test tube. These tubes should be capped and kept cold out of intense light until photometric analysis for oxyhemoglobin. This is done by determination of optical density at 541 mμ or at 412 mμ, depending on sensitivity required. With a reaction mixture containing 2 x 10^8 cells per ml., and a sampling dilution of 1/3, complete lysis will yield an optical density of about 0.7 at 541 mμ. At 412 mμ the sensitivity is approximately nine times greater, and therefore, this wavelength should be used for analyses with low cell concentrations or high sampling dilutions. It is also advantageous that the absorption spectra of oxyhemoglobin and methemoglobin intersect at 412 mμ. Different Beckman DU spectrophotometers have been found to vary slightly in this respect, and it is advisable to ascertain the point of intersection for each instrument.

Three or four operators are needed for efficient conduct of this type of kinetic experiment. One person conducts the sampling, another handles the stop diluent tubes, as needed by the sampling operator, and a third person operates the centrifuges. The fourth operator is needed for record keeping and timing during the sampling operations, but a tape recorder can be substituted.

"Ghost Sampling"

Photometric analysis of the supernatant fluid obtained upon immediate centrifugation after withdrawal of a sample, yields a measure of the number of cells hemolyzed, or the number of ghosts formed, at the time of sampling. Kinetic curves obtained in this manner are referred to as "ghost" curves (23), in order to distinguish them from E* curves. The usual kinetic hemolysis curves, such as those shown in Figures 37-42 are "ghost" curves.

E* Sampling

For E* analyses, samples are withdrawn as described above and centrifuged thoroughly. The supernatant fluid is poured off and analysed for hemoglobin. The button of sedimented cells obtained on centrifugation is drained thoroughly free of fluid by inversion of the tube on a clean towel, or a piece of filter paper, the lip is wiped and the cells are suspended in an accurately measured volume of diluent (usually 3 to 5 ml.). The tubes are then incubated for one hour at 37°C. for the transformation of E* to ghosts (this applies to guinea pig complement; in the case of human or monkey complement, as long as eight hours' incubation is not quite adequate for complete E* to ghost conversion, and therefore, in these cases only approximate E* determination can be made.) At the end of the incubation period the tubes are centrifuged and the supernatant fluids analyzed photometrically for hemoglobin in the usual way. Even with careful draining after removal of the initial supernatant fluid, a trace of this fluid is retained with the sedimented button of cells and an appropriate correction should be applied for the hemoglobin, if any, contributed to the E* measurement through this carry-over. If measurements of "E* plus ghosts" are desired, sum up the hemoglobin contents of the original supernatant fluid and of the supernatant fluid from the cell button after the additional one-hour incubation.

Before discovery of the terminal transformation reaction, it had been customary to collect samples through the entire kinetic run and to store them in the cold for centrifugation at the end of the experiment. In view of the fact that formation of ghosts from E* proceeds even at 0°C., this practice yields a kinetic analysis intermediate between that obtained by "ghost sampling" and "E* + Ghost Sampling," as illustrated in Figure 64 for the reaction between EA and guinea pig complement.

In earlier publications from this laboratory, a simplified technic of E* sampling was described, in which samples were mixed with several volumes of ice-cold buffer and centrifuged. After sedimentation of the cells the tubes were withdrawn carefully from the centrifuge so as to avoid disturbing the button of cells and placed in a water bath at 37°C. for about one hour *without* agitation. Since the cells were kept in the sedimented state during the second period of incubation they were out of effective contact with the bulk of the fluid phase containing C'3, and therefore, no appreciable further formation of E* occurred. This short-cut procedure was found acceptable in analyses involving a high cell concentration (2×10^8 per ml.), but in studies of the action of C'3 on EAC'1, 4, 2 in which it is necessary to use a low cell concentration (3×10^7 per ml.) in order to minimize consumption of C'3, this simplified technique has been found invalid since action of C'3 on the sedimented cells during the second incubation

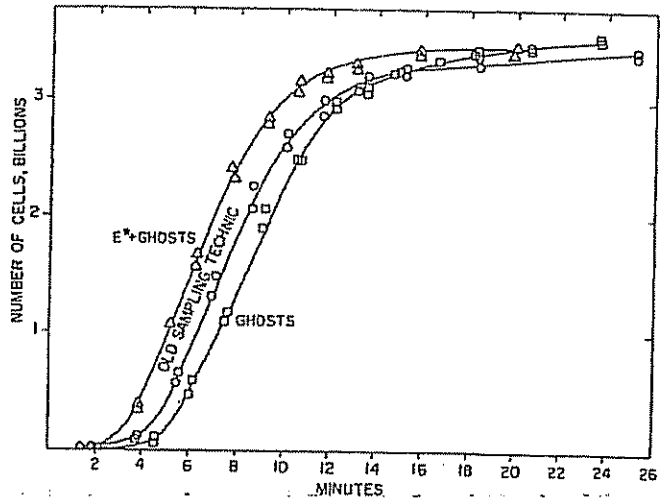

FIG. 64. Kinetics of the limited complement system as studied by three different sampling technics. The curve marked "ghosts" is obtained by immediate centrifugation of samples and spectrophotometric analysis of the supernatant fluids for oxyhemoglobin. The curve marked "E* + ghosts" is obtained by collection of samples in several volumes of ice-cold citrate-saline followed by centrifugation. The sample tubes are then incubated at 37°C. for about one hour without disturbing the button of sedimented cells. During this time all of the cells in the state E* at the moment of sampling release their hemoglobin. Following incubation the sample tubes are chilled, their contents are mixed, and after centrifugation the oxyhemoglobin content of the supernatant fluids is measured, yielding a determination of the sum of E* and ghosts at the moment of sampling. The intermediate curve, labelled "old sampling technique," is obtained by collection of samples in ice-cold citrate-saline, followed by centrifugation and analysis of the supernatant fluids upon completion of the entire experiment. The position of this intermediate curve may vary depending on the period of time elapsed between collection of the samples and centrifugation (modified from ref. 267).

is not arrested completely, and as a consequence, erroneously high E* values are obtained (cf. Fig. 63).

Kinetic Analysis of Conversion of EAC'1, 4, 2 to E* by C'3

EAC'1, 4, 2 are prepared as described in the next section. In view of the rapid decay of this intermediate product at elevated temperatures, it is essential to maintain the cell suspension at 0°C. up to the moment of initiation of the reaction. Therefore, it is not feasible to follow the usual practice of charging the Erlenmeyer flasks with the cell suspension and buffer, followed by addition of complement eight or ten minutes later when the contents of the reaction flask have reached the temperature of the incubation bath. Instead, 24 ml. of an appropriate dilution of guinea pig serum in gelatin-buffer containing 0.005 *M* EDTA are pipetted into the reaction flask (this mixture serves as a source of C'3; mixtures of purified C'3a and C'3b, if available, may be used instead). After several minutes for equilibration to temperature, 1.0 ml. of ice-cold EAC'1, 4, 2 (7.5×10^8 cells per ml.) is injected quickly and with agitation to achieve prompt and uniform mixing. It is convenient to use an accurately calibrated 1 ml. tuberculin syringe for delivery of the cells. In this fashion a series of reac-

tion flasks can be set up with EAC'1, 4, 2 of the same reactivity to C'3, i.e., the same number of $SA_2C'1$, 4, 2 per cell. Sampling is performed according to the E^* procedure, as described above, with a sampling dilution of 1/10 and prompt centrifugation. A larger sampling dilution, though desirable is not feasible because optical density readings would be too low, even at the sensitive wavelength of 412 mμ. Unfortunately, in this type of analysis one is forced to compromise between the conflicting requirements of low cell concentration and high sampling dilution. The use of Cr^{51} labelled cells might improve the procedure, but this has not yet been explored for this purpose.

Kinetic Analysis of the Formation of EAC'1, 4, 2 from EAC'1, 4 by Action of C'2

EAC'1, 4 are prepared as described in the following section. Reaction flasks are charged with 10.0 ml of cell suspension (1.5×10^8/ml) in gelatin-buffer containing 0.0005 *M* Mg^{++} and 0.00015 *M* Ca^{++}. After rocking for about ten minutes to attain temperature, 10.0 ml. of an appropriate dilution of purified C'2, also in buffer containing 0.0005 *M* Mg^{++} and 0.00015 *M* Ca^{++}, are added and timing is started. At suitable intervals, 1.0 ml. samples are withdrawn and delivered immediately into 1.5 ml. of an appropriate dilution of guinea pig serum in buffer containing 0.005 *M* EDTA. (A dilution of 1/37.5 has been found suitable.) The contents are mixed immediately and the tubes are incubated for about ninety minutes at 37°C. with occasional manual agitation to maintain the cells in uniform suspension. At the end of this period, 5.0 ml. of 0.15 *M* NaCl are added and the contents are mixed. The tubes are then centrifuged and the supernatant fluids are analyzed for oxyhemoglobin at 412 mμ. Complete lysis corresponds to an optical density of about 0.9 at this wavelength.

A crucial element in these analyses is the choice of an appropriate dilution of C' in EDTA-buffer as a source of C'3. In view of the decay of EAC'1, 4, 2, the efficiency of conversion of this intermediate to E^* depends on the concentration of C'3. Complete conversion is not feasible with the reagents available at present, but as shown in Fig. 65, this goal can be approached quite closely with C' dilutions

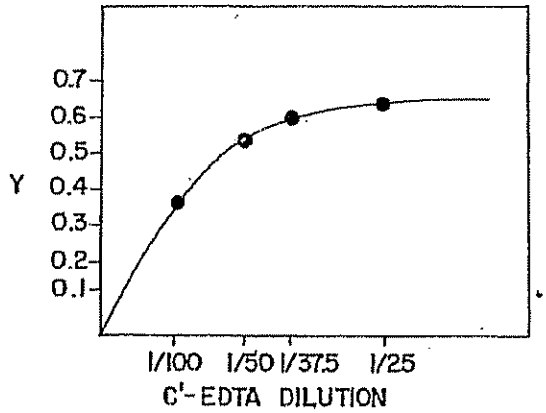

FIG. 65. Effect of variation of C'3 (C'—EDTA) concentration on the extent of lysis of EAC'1, 4, 2 formed from EAC'1, 4 with a constant amount of C'2. C'-EDTA was added at peak-time of EAC'1, 4, 2 formation.

of 1/37.5, or lower. In most experiments of this type, a 1/37.5 dilution has been used because lower dilutions sometimes gave rise to erratic cell blank values.

Kinetic Analysis of the Conversion of EAC'1 to EAC'1, 4 by C'4

While detailed studies of the kinetics of this reaction are not yet available, from unpublished experiments by L. G. Hoffmann a general outline of procedure can be given. EAC'1 are made from EA with chromatographically separated guinea pig C'1; alternatively, the procedures of Laporte *et al.* (79) or Klein (139) can be used. Ten ml. of EAC'1 (10^9 cells per ml.), in buffer containing 0.001 *M* Ca^{++}, are mixed with an equal volume of chromatographically separated C'4 (152), also in buffer containing 0.001 *M* Ca^{++}. At suitable intervals, 0.5 ml. samples are withdrawn, mixed with 7.0 ml. of ice-cold diluent and centrifuged immediately in the cold to arrest the action of C'4. (Veronal buffer is used as stop-diluent for these analyses; EDTA-buffer is not suitable because it causes loss of activity, presumably due to dissociation of C'1.) The supernatant fluid from each sample is poured off, the tube is allowed to drain, the lip is wiped and the sedimented cells are suspended in 2.0 ml. of buffer containing 0.001 *M* Mg^{++} and 0.00015 *M* Ca^{++}. Three 0.50 ml. portions from this suspension are pipetted into separate test tubes; one of these will serve as a cell stability control, the second will be lysed completely with 0.1% sodium carbonate to check the cell concentration, and the third will be treated with 0.50 ml. of purified C'2 at a concentration chosen so that about 200 effective molecules of C'2 per cell will be supplied. This quantity suffices to convert all $SA_2C'1, 4$ to $SA_2C'1, 4, 2$. The treatment with C'2 is performed at 30°C. for a period of thirty minutes. Next, 1.5 ml. of a 1/37.5 dilution of guinea pig complement in 0.005 *M* EDTA-buffer are added. After ninety minutes' incubation at 37°C., 5.0 ml. of 0.15 *M* NaCl are added, the contents are mixed, the tubes centrifuged and the supernatant fluids analyzed for hemoglobin at 412 mμ.

The degree of lysis, y, or alternatively, $-\ln(1-y)$ is plotted against time. It has been found that formation of EAC'1, 4 starts immediately on addition of C'4, i.e., there is no lag, and the curve rises, with continuously diminishing slope, until the maximum is reached. This requires about ten minutes, and the level of $SA_2C'1, 4$ remains constant thereafter for at least one hour.

Kinetic Flow Analysis

This term refers to studies of the reaction of EA with C' in which successive samples withdrawn from the reaction mixture are treated in rotation by the several sampling techniques which have been described, namely, ghost sampling, E* sampling, EAC'1, 4, 2 sampling and EAC'1, 4 sampling. In this fashion, the formation and disappearance of each of these intermediate products may be visualized in a single experiment. The following procedure is taken from (157): A flask containing 6×10^9 EA and 1.0 ml. of a 1/14 dilution of C' in a final volume of 90 ml. with 0.00015 *M* Ca^{++} and 0.0005 *M* Mg^{++} present, is rocked continuously in a water bath at 37°C. Samples of 1.5 ml. are removed at the desired intervals and mixed with 3.0 ml. of ice-cold veronal buffer. They are centrifuged in the cold for two to five minutes at 1000 g promptly after withdrawal. Supernatant fluids are removed immediately after centrifugation and set aside for hemoglobin analysis (ghost sampling); the tubes are drained and the lips wiped with filter paper. The sedimented cells are suspended smoothly in *one* of the following reagents: 1 ml. C'y diluted 1/24 with respect to original serum in veronal buffer containing 0.001 *M* Mg^{++} (EAC'1, 4 sampling), 1.0 ml. C'y diluted 1/24 with respect to original serum in veronal buffer containing 0.005 *M* EDTA (EAC'1, 4, 2

sampling), or 5.0 ml. of plain veronal buffer (E* sampling). To retard further reactions of C′ in the samples after withdrawal and during these manipulations, the entire experiment is performed in a cold room. The resuspended cells are further incubated for one hour at 37°C. Then, 3.5 ml. of veronal buffer is added to those tubes which had previously received C′y. All tubes are then centrifuged and the supernatant fluids are diluted 1/3 for hemoglobin determination at 412 mμ.

C′y was used in (157) because purified C′2 was not available at the time. Since the preparation of C′y is quite laborious, it would be preferable to use purified C′2 for the EAC′1, 4 sampling, but in this event the conversion of EAC′1, 4 to E* will have to be performed in two successive steps; first, treatment with C′2 and second, treatment with EDTA-C′, as described above.

An interesting application of the kinetic flow method has been made by Rodriguez and Osler (158) in a study of the interference of phlorizin with the hemolytic action of complement. From their results, shown in Figures 66 and 67, it is evident that phlorizin interferes in the reaction of EAC′1, 4, 2 with C′3. Thus, it is evident from both graphs that EAC′1, 4 reached a peak within one or two minutes following addition of C′, and peak formation of EAC′1, 4, 2 required about five minutes, in both cases. In the absence of phlorizin (Fig. 66), this was followed by rapid decline of EAC′1, 4, 2 due to interaction with C′3 leading to formation of E*, which peaks in about ten to twelve minutes. By fifteen minutes E* began to decline due to transformation to ghosts.

In the presence of phlorizin (Fig. 67), EAC′1, 4, 2 did not disappear rapidly and no E* or ghosts were formed. Presumably, phlorizin interfered with the transformation of EAC′1, 4, 2 to E* by action of C′3. This substantiates the conclusion reached earlier by Mills and Levine (159) with respect to inhibition by salicylaldoxime.

In the interpretation of curves such as those shown in Figures 66 and 67, it should be borne in mind that the ordinates do not indicate the absolute number of cells in the condition of EAC′1, 4 or EAC′1, 4, 2. Instead, these ordinates are relative in-

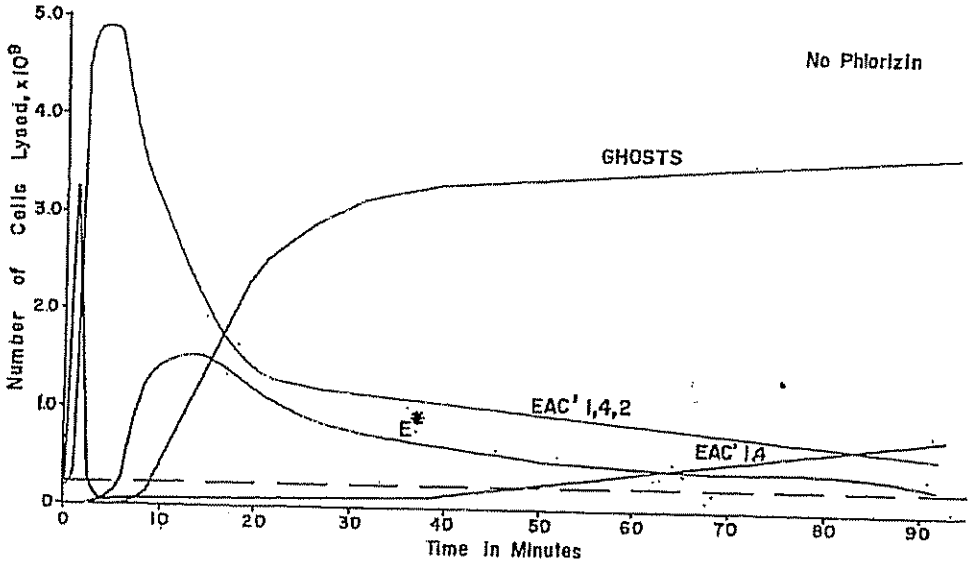

FIG. 66. Kinetic flow analysis of the reaction between EA and C′. From (158).

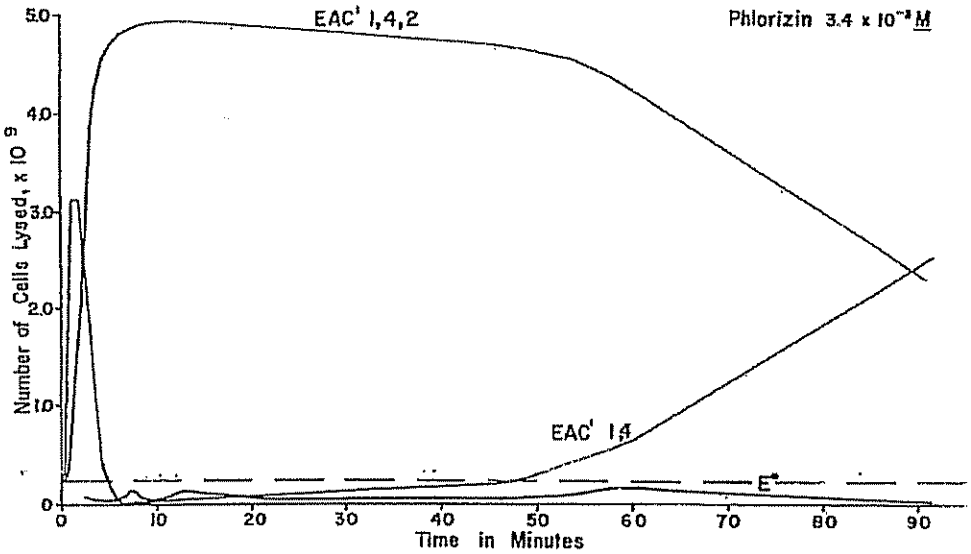

FIG. 67. Kinetic flow analysis of the reaction between EA and C′ in the presence of phlorizin. No E* or ghosts are formed due to blocking of C′3 action. From (158).

dices of EAC′1, 4 or EAC′1, 4, 2 *reactivity*, reflecting the availability of $SA_2C'1, 4$ or $SA_2C'1, 4, 2$ in the reaction mixtures. On the other hand, with respect to E* and ghosts the ordinates indicate the absolute number of cells in these conditions.

EXPERIMENTAL PROCEDURES FOR PREPARATION OF INTERMEDIATE PRODUCTS

Preparation of EAC′1, 4

The original procedure (132) required the use of a reaction system containing Ca^{++}, but not Mg^{++}. This was obtained by removal of divalent cations through treatment with ion exchange resin, followed by addition of Ca^{++}. Since the discovery that $SA_2C'1, 4, 2$ decays to $SA_2C'1, 4$ on incubation at 37°C., the strict exclusion of Mg^{++} is no longer necessary and a simpler procedure can be given, as follows:

Ten ml. of optimally sensitized erythrocytes, 1 x 10^9 cells per ml., in veronal-gelatin buffer containing 0.001 *M* Ca^{++}, are mixed with 0.5 to 1 ml. of guinea pig serum and allowed to react, strictly at 0°C., with occasional agitation, for thirty to ninety minutes, as determined in preliminary experiments for each lot of guinea pig C′. The optimal quantity of complement may vary somewhat with different lots of guinea pig serum and should be determined by trial. The use of more than 1 ml. will not enhance reactivity appreciably, and may even be inhibitory. (If EAC′1, 4 of low reactivity is desired, the quantity of complement should be decreased, of course.) The time of treatment should be adjusted so that the extent of formation of E* does not exceed about 5%. After treatment, the cells are centrifuged, the supernatant fluid is removed, and the cells are washed twice with ice-cold diluent. They are then suspended in 20 ml. of diluent and incubated for ninety minutes at 37°C. The suspension is then centrifuged, the supernatant fluid removed and its optical density is measured in order to determine the percentage of cells which went to the state E* during

the initial treatment of EA with C′. The cells are resuspended in diluent to the desired concentration.

Washed EAC′1, 4 are stable and may be stored at 0°C. for several days in the usual gelatin-veronal buffer containing 0.0005 *M* Mg^{++} and 0.00015 *M* Ca^{++}. For use, wash once with this diluent and adjust cell concentration as desired.

Comments

(1) For sensitization of erythrocytes, Forssman antibody (rabbit antibody to boiled sheep erythrocyte stromata) should be used. The applicability of this procedure to other types of hemolysin has not been evaluated.

(2) Some lots of guinea pig C′ may not yield EAC′1, 4 of good reactivity. If difficulty is encountered, try a different lot of C′.

Preparation of EAC′1, 4, 2

There are three methods. The original procedure (133) involves treatment of 10 ml. of optimally sensitized erythrocytes, 1 x 10^9/ml., with 1 ml. of undiluted guinea pig serum for about twenty to thirty minutes at 0°C. in the presence of 0.0005 *M* Mg^{++} and 0.00015 *M* Ca^{++}. The second method (152) makes use of blocking the action of C′3 with formaldehyde. The third procedure, described below, as the method of choice, is based on conversion of EAC′1, 4 to EAC′1, 4, 2 by purified C′2. Its advantages are: (1) No E* formation and therefore no need to store the cells for four hours at 0°C. prior to use. (2) The number of $SA_2C'1$, 4, 2 per cell can be adjusted at will by variation of the quantity of C′2.

Ten ml. of EAC′1, 4 (1 x 10^9 cells per ml.) in diluent containing 0.00015 *M* Ca^{++} and 0.0005 *M* Mg^{++}, are mixed with 10 ml. of purified C′2, furnishing about 100 to 200 effective molecules of C′2 per cell, and incubated at 30°C. for the period of time required for peak formation of $SA_2C'1$, 4, 2. This depends on the concentration of $SA_2C'1$, 4 and should be determined experimentally for each lot of EAC′1, 4. At the end of this period, add 3 to 4 volumes of ice-cold diluent, immerse tube in ice-water, centrifuge at once in the cold, and suspend cells in 10 ml. of ice-cold diluent, either the usual Ca^{++} Mg^{++} buffer, or EDTA-buffer, depending on the requirements of the experiment. Store at 0°C. until needed. Use promptly, since decay occurs even at 0°C. (half-life about 10 hours).

Preparation of EAC′1

1. Preparation with Ether-Treated Guinea Pig Complement According to Klein *(139)*: One-hundred ml. of ethyl ether (USP solvent ether) are placed in a 500 ml. Erlenmeyer flask and warmed to 30°C. in a water bath on a magnetic stirrer. Under high speed stirring 4 ml. of guinea pig serum are added. The mixture is stirred for about five minutes, the ether is then rapidly decanted and the remnants of ether removed by applying a gentle stream of compressed air into the flask for a few minutes. The ether-treated guinea pig serum is then diluted 1/10 with ice-cold diluent containing 0.001 *M* Ca^{++}. Store in ice-water.

Five ml. of EA (5 x 10^8 per ml.) suspended in buffer containing 0.001 *M* Ca^{++}, are warmed to 37°C. and mixed with 5 ml. of the 1/10 dilution of ether-treated complement also pre-warmed. The mixture is incubated at 37°C. for ten minutes. Centrifuge and wash cells twice with 10 ml. of buffer containing 0.001 *M* Ca^{++}. Suspend in Ca^{++}-buffer to a concentration of 10^9 cells per ml. and store at 0°C.

Comments

Longer treatment may be required if anesthesia ether is used. This observation suggests that an impurity in ether participates in the reaction.

2. Method of Hoffmann *(140)*: Set up a chromatographic column of 8 cm. inside diameter and 60 cm. length containing

25 gm. DEAE-cellulose in 0.08 *M* NaCl at pH 7.4 (cf. preparation of purified C'2, C'3a and C'3b). The column is washed with 0.08 *M* NaCl until the effluent is free of fine particles. Twenty ml. of guinea pig serum are diluted with 17.5 ml. of distilled water and the mixture is applied to the column. After the serum has entered the column (do not allow column to run dry), 0.08 *M* NaCl is added. Apply pressure with a squeeze bulb to speed up flow. The first 100 ml. of effluent are discarded. Collect the second 100 ml. portion which should contain approximately 45% of the total serum proteins, as determined photometrically at 280 mμ. This effluent is immediately adjusted to an ionic strength of 0.3 by addition of 3 *M* NaCl solution. The fraction is stored at −30°C. and will keep for about one month.

For preparation of EAC'1, dilute this fraction ½ with distilled water to adjust ionic strength to 0.15. Forty ml. of EA (5×10^8 per ml.), suspended in buffer containing 0.001 *M* Ca^{++}, are mixed with 10 ml. of the ½ dilution of the 0.08 *M* effluent (this quantity is usually sufficient to make a EAC'1 preparation yielding maximal activity with C'4; this should be checked experimentally, and, if necessary, a larger amount should be used for treatment of EA). Incubate the mixture for ten minutes at 37°C., centrifuge, wash twice with about 50 ml. of 0.001 *M* Ca^{++}-buffer and suspend the cells to a concentration of 10^9 per ml. in Ca^{++}-buffer. Store at 0°C. Preparations of EAC'1 can be kept for two days, but should be washed with Ca^{++}-buffer just prior to use. For improved procedure consult ref. 269.)

TITRATION OF COMPLEMENT COMPONENTS WITH INTERMEDIATE PRODUCTS AS SUBSTRATES

Titration of C'2

Prepare a suspension of EAC'1, 4 containing 1.5×10^8 cells per ml. in diluent containing 0.00015 *M* Ca^{++} and 0.0005 *M* Mg^{++}. Pipette 0.50 ml. portions into a series of 15 x 125 mm. test tubes and place in a water bath at 30°C. ± 0.02°. Prepare an accurate series of two-fold dilutions of C'2 in diluent containing 0.00015 *M* Ca^{++} and 0.0005 *M* Mg^{++}, and warm to 30.0°C. Add 0.50 ml. of each dilution of C'2 to a tube of cells and mix contents. The additions of C'2 should be staggered at uniform intervals of 0.5 or one minute. Incubate for the period of time, t_{max}, required for optimal $SA_2C'1, 4, 2$ formation at this cell concentration. (t_{max} depends on the product of the cell concentration and the number of $SA_2C'1, 4$ per cell.) This should be determined in a preliminary experiment for each lot of EAC'1, 4; if t_{max} exceeds eighteen minutes, the number of $SA_2C'1, 4$ per cell is too low for satisfactory titration of C'2.

At the end of the proper incubation period, add 1.5 ml. of guinea pig serum diluted 1/37.5 in diluent containing 0.005 *M* EDTA. These additions should be staggered at the same intervals as the C'2 additions in order to maintain uniform timing. Immediately after addition of C'-EDTA, transfer the tube to a water bath at 37°C ± .02. After ninety minutes incubation with occasional agitation, add 5.0 ml. of 0.15 *M* NaCl to each tube, mix, centrifuge, decant supernatant fluid and read at 412 mμ.

In addition to the titration proper, set up a control of EAC'1, 4 + C'-EDTA. A tube yielding complete lysis should be set up with 50 to 100 effective molecules of C'2. In addition, the optical density of the C'-EDTA should be read at 412 mμ and results corrected accordingly.

In order to calculate the effective number of C'2 molecules, plot $-\ln(1-y)$, where y is the fraction of cells lysed, against the reciprocal of the dilution (relative concentration) of C'2. This should yield a straight line. The relative concentration

of C′2 at which $-\ln(1-y)$ = unity is then read from the graph. The reciprocal of this value is multiplied by 2 (because 0.5 ml. of C′2 dilution was used) and by the number of cells (7.5×10^7) to obtain C_{max}, the number of $SA_2C'1, 4, 2$ at t_{max}. B_o, the absolute number of effective C′2 molecules is calculated with equation [3] given in the theoretical section.

The value of k_2 needed for this calculation is obtained from a decay experiment (usually $k_2 = 0.030$ min.$^{-1}$ at 30°C.). The value of k_1A_o is obtained from t_{max} by use of equation [8] in the theoretical section. For convenience of calculation, use the values in the following tabulation, which are applicable if $k_2 = 0.030$ min.$^{-1}$.

t_{max}	$\frac{k_1A_o}{k_1A_o - k_2}\left(e^{-k_2t_{max}} - e^{-k_1A_ot_{max}}\right)$
8	0.78
10	0 74
15	0.64
20	0.55

Example

Let us assume that 0.5 ml. of a 1/460 dilution of a given preparation of C′2 yielded $-\ln(1-y) = 1$, i.e., $y = 0.63$ on reaction with an $EAC'1, 4$ preparation having a t_{max} of fifteen minutes. The C′2 preparation contains $460 \times 2 \times 7.5 \times 10^7 \times \frac{1}{.64} = 1.08 \times 10^{11}$ effective molecules of C′2 per ml.

Comments

(1) The term "effective" as used in this connection, means that the C′2 value represents a minimal estimate, since the possibility of unfruitful reaction resulting in destruction of C′2 cannot be excluded.

(2) The applicability of this method to the titration of C′2 in whole serum has not yet been evaluated fully.

Titration of C′3

These measurements are made with 7.5×10^7 cells in a total reaction volume of 2.5 ml. The experimental conditions are the same as those described for kinetic analyses of C′3 action on $EAC'1, 4, 2$, except that the reaction is carried to the endpoint, which requires about ninety minutes at 37°C. The titers so obtained are only *relative*, since they depend on the concentration of $SA_2C'1, 4, 2$. The response curve is sigmoidal and the von Krogh equation or the probit function may be used to estimate the 50% reference point on which the titer is based.

Titration of C′3a or C′3b

These factors are titrated in the same manner, except that one of the factors is kept constant. The titer of C′3a depends on the concentration of C′3b, and conversely, the titer of C′3b depends on the concentration of C′3a.

Titration of C′4

Prepare a suspension of EAC′1 containing 10^9 cells per ml. and a series of accurate dilutions of C′4 in diluent containing 0.001 *M* Ca^{++}. In a bath kept at 0°C., mix 1.0 ml. portions of EAC′1 with 1.0 ml. of each C′4 dilution. Set up a cell stability control (these cells receive no C′ reagents), a "C′2, 3 blank" (these cells receive no C′4), and a "complete" (use excess C′4). Incubate for twelve minutes at 37°C. and add 4.5 ml. of diluent containing 0.001 *M* Mg^{++}, to each tube. Immediately pipette 0.5 ml. of each suspension into a tube containing 0.5 ml. of purified C′2, diluted in buffer containing 0.001 *M* Mg^{++} so as to supply about 100 effective molecules per cell. These mixtures are incubated for at least

thirty minutes at 30.0°C. with occasional manual agitation. The large multiplicity of C'2 and the long incubation period are required to convert every $SA_2C'1$, 4 to $SA_2C'1$, 4, 2, because at the low level of $SA_2C'1$, 4 (less than 5 sites per cell) employed, the rate of formation of $SA_2C'1$, 4, 2 is slow compared to its decay. The exact time of incubation is not critical, however, since sufficient C'2 remains in the fluid phase to maintain a steady state between formation and decay of $SA_2C'1$, 4, 2 for over one hour.

After incubation at 30°C., each tube receives 1.5 ml. of whole C', diluted 1/37.5 in diluent containing 0.005 *M* EDTA. Transfer at once to a water bath at 37°C. and incubate for ninety minutes with occasional manual agitation. Then add 5.0 ml. of 0.15 *M* NaCl, centrifuge, collect the supernatant fluids and measure hemoglobin at 412 mμ. Determine extent of lysis, y, and plot $-\ln(1-y)$ arithmetically against the reciprocal of the C'4 dilution. This should yield a straight line if the EAC'1 preparation employed contains a sufficient number of $SA_2C'1$ per cell to permit random distribution of the C'4 among the cells. The titer is expressed in terms of effective molecules of C'4, as in the titration of C'2.

The method is applicable to whole serum. Pooled guinea pig serum contains 3 to 4 x 10^{12} effective molecules of C'4 per ml.

ISOLATION AND PURIFICATION OF COMPLEMENT COMPONENTS

Pillemer, Ecker, Oncley and Cohn (160) applied the salting-out methods developed by Cohn and his collaborators (161) to the fractionation of guinea pig complement. C'1 activity was entirely contained in the fraction of serum protein insoluble in 1.39 *M* $(NH_4)_2SO_4$, C'3 was the only component present in the fraction soluble in 1.39 *M* $(NH_4)_2SO_4$, but insoluble in 2.0 *M* $(NH_4)_2SO_4$, and C'2 and C'4 were soluble in 2.0 *M* but insoluble in 2.5 *M* $(NH_4)_2SO_4$.

Preliminary to fractionation the complement-containing serum was freed from fat by centrifuging in the cold. C'1 was then precipitated by dialysis in the cold against phosphate buffer of pH 5.2 and ionic strength 0.02. The precipitate was redissolved and brought to 1.22 *M* $(NH_4)_2SO_4$, centrifuged, and the precipitate redialyzed against the phosphate buffer. This purified fraction retained full C'1 activity and was homogeneous in the ultracentrifuge and 98% homogeneous by electrophoresis. The yield of material represented 0.6% of the total serum protein. Chemical and physical properties of purified C'1 are given in Table 10.

Eighty to 85% of the C'2 and C'4 components were contained in the material soluble in 2.0 but insoluble in 2.2 *M* $(NH_4)_2SO_4$. On dialysis against distilled water the two components precipitated as a green transparent viscous sediment which was washed with distilled water and dissolved in 0.9% NaCl. The $(NH_4)_2SO_4$ precipitation and dialysis were repeated several times. This "muco-euglobulin" contained 85% of both components C'2 and C'4. Its properties are given in Table 10.

A method for the separation and purification of C'1 from human serum has been described by Pillemer *et al.* (162). Human C'1 was characterized as a euglobulin with an electrophoretic mobility of 2.9 x 10^{-5} in veronal buffer of pH 7.8 and ionic strength 0.1. The material showed two components in the ultracentrifuge. The principal component, comprising 70% of the material, had a sedimentation constant of 6.9 when converted to water at 20°C. The remaining 30% of the preparation sedimented more rapidly.

More recent studies on the purification of C'1 from human serum have been made by Lepow *et al.* (115), in connection with investigation of the esterase problem. One-hundred ml. of human serum or RP

TABLE 10

Properties of Purified Components of Guinea Pig Complement

	Euglobulin	Muco-euglobulin
Complement components present	Mid-piece	End-piece and 4th component
Mobility in phosphate buffer, at pH 7.7, 0.2 ionic strength	-2.9×10^{-5}	-4.2×10^{-5}
Sedimentation constant 1%, 20°, W	-6.4×10^{-13}	—
Protein nitrogen, per cent	16.3	14.2
Carbohydrate, per cent	2.7	10.3
Phosphorus per cent	0.1	0.1
Optical rotation, $°[\alpha]_d^{25°}$	−28.7	−192.6
Apparent isoelectric point	5.2—5.4	6.3—6.4
Fraction of total complement-activity, per cent	100	85
Fraction of total serum-protein, per cent*	0.6	0.18
Heat stability of complement activity (destroyed in 30 mins. at tabulated temperature), °C.	50	50† 66‡

*The three complement components together then comprise 0.78 per cent of the total serum-protein.
†For end-piece activity.
‡For 4th component activity.
Data from (160).

are dialyzed against pH 5.5 acetate buffer, $\mu = 0.02$, at 1°C. for thirty-six to forty-eight hours. The precipitated C′1 is recovered by centrifugation at 1°C., washed twice with 100 ml. of dialysate, and extracted at 1°C. for one hour with 200 ml. pH 5.5 acetate buffer, $\mu = 0.12$. The remaining precipitate is re-extracted at 1°C. for one hour with 100 ml. pH 5.5 acetate buffer, $\mu = 0.50$. This extract is dialyzed overnight against 2 liters of pH 5.5 acetate buffer, $\mu = 0.20$ at 1°C. The supernatant fluid after centrifugation is brought to 10% methanol at 0 to −2°C, and the resulting precipitate suspended in 0.3 *M* NaCl to a final volume of 5 ml. and centrifuged in the Spinco preparative ultracentrifuge at 1°C., 100,000 g for one hour. The slightly opalescent supernatant fluid, at pH 5.5, $\mu = 0.30$, is stored at −30°C.

The final fraction contains 17 to 25% of the C′1 in the original serum, with a purification of 30 to 50-fold. The preparation has been found to be heterogeneous in the ultracentrifuge, showing at least three components at pH 5.5, ionic strength 0.30.

This partially purified preparation of C′1 was not anticomplementary, unlike the preparations obtained by the Pillemer method. It contained no measurable C′2 or C′4, as detected with R2 and R4, respectively, but traces of C′3, as detected with R3, were present. In preparations made from human serum, properdin was present. In addition, the material contained appreciable amounts of streptokinase-activable plasminogen, and traces of thrombin and perhaps prothrombin in some preparations. Hageman factor was present in every preparation.

As pointed out before, C′1 also can be obtained by elution from EAC′1, 4 or EAC′1, 4, 2 with EDTA (79, 80), but preparations of this type have not yet been characterized in detail.

Jonsen *et al.* (143) have used precipitation with alcohol for separation of C′3 from pig serum. In recent studies by Arday, Pillemer and Lepow (154) purification

of C'3 from human serum has been described. In view of the dual nature of C'3 and the lack of adequate information as to which of the C'3 factors is lacking in R3, the reagent used in the studies by Jonsen and Arday, the interpretation of these investigations is uncertain.

The initial separation of C'3a and C'3b by Rapp (90) was made by means of acid and alcohol precipitation, respectively. An improved method of purification by chromatography on DEAE-cellulose has been developed recently and will be given below.

Chromatographic Separation of the Complement Components

Becker (163), as well as Rapp, Sims and Borsos (152), demonstrated the feasibility of separation of C' components by chromatography on cellulose derivatives. Further studies by Rapp and Borsos (164) have led to the development of rapid and simple procedures, involving chromatography under pressure or vacuum to achieve a high flow rate, and utilizing step-wise rather than gradient elution. Procedures of this type for separation of C'2, C'3a and C'3b from guinea pig serum will be presented.

Separation of C'2 from Guinea Pig Serum (according to Borsos)

Thirty gm of reagent grade DEAE-cellulose* are placed in a 2000 ml. beaker and suspended in approximately 1200 ml. of 0.5 *M* NaCl. The mixture is stirred well by means of a large magnetic mixer and poured into a closed sintered glass filter of coarse porosity. After draining thoroughly under pressure, by a squeeze bulb, the cellulose is resuspended in 1200 ml. of 0.5 *M* NaCl and washed again. After a third wash with 0.5 *M* NaCl, the cellulose is washed with 1200 ml. 0.08 *M* NaCl and suspended in 1200 ml. 0 08 *M* NaCl. The pH of the suspension is adjusted to 7.4 by addition of 1 *N* HCl.

Twenty gm. of CM-cellulose are washed three times with 1 liter of 0.5 *M* NaCl, as above, followed by one wash with 1 liter of 0.03 *M* NaCl. The cellulose is then suspended in 800 ml. of 0.03 *M* NaCl and the pH is adjusted to 4.6 with 1 *N* NaOH.

For isolation of C'2, three chromatographic columns are set up. Column 1 is a tube of 8 cm. inside diameter and 60 cm. length, containing 20 gm. DEAE-cellulose in 0.08 *M* NaCl at pH 7.4. The height of the cellulose column should be about 5 cm. In packing the column, a plug of glass wool is inserted at the bottom and moistened with distilled water before pouring the cellulose slurry into the tube. After the cellulose has settled (apply pressure with a squeeze bulb), another plug of glass wool is placed on top of the cellulose. Glass beads are poured on top of the glass wool to keep it in place and to maintain the column in the properly packed state. The column is then washed with 0.08 *M* NaCl until the effluent is free of fine particles (1 or 1½ liters usually required).

Column No. 2 contains 20 gm. of CM-cellulose at 0.03 *M* NaCl and pH 4.6 in a tube of 5 cm. inside diameter and 60 cm. length. The height of the column should be about 7.4 cm. Place glass wool and beads on top of the cellulose and wash with 0.03 *M* NaCl until effluent is clear.

Column No. 3 contains 7.5 gm. DEAE-cellulose at 0.015 *M* NaCl and pH 7.4 in a tube of 2.5 cm. inside diameter and 60 cm. length. The height of the column should be about 5.5 cm. Place glass wool and beads on top of the cellulose and wash with 0.015 *M* NaCl until effluent is clear.

Twenty ml. of cold guinea pig serum are adjusted to pH 5.6 by careful addition of 0.15 *M* HCl delivered under the surface with continuous stirring. Dialyze against 1000 ml. of cold distilled water with continuous stirring until the ionic strength inside the bag is 0.01. (This is determined

*This procedure is applicable to the DEAE and CM-cellulose available from Brown Company, Berlin N. H.

by periodic checks of the conductivity of the fluid outside the dialysis bag.) Remove serum and centrifuge in the cold for ten minutes at about 750 g. Readjust ionic strength of the clear supernatant fluid to 0.08 with NaCl; the pH is brought back to 7.2 with careful dropwise addition of 0.08 *M* NaOH under continuous stirring and with the tip of the pipette extending below the surface of the serum. This adjusted supernatant fluid is poured into column No. 1, and pressure is applied with a squeeze bulb to hasten the passage of the serum through the cellulose. As soon as all of the serum has entered the cellulose column, 500 ml. of 0.08 *M* NaCl are added (do not allow top of column to become dry). Discard the first 100 ml. of effluent and then collect the next 300 ml. in a graduated cylinder containing 500 ml. of ice-cold distilled water. The 800 ml of mixture so obtained are well mixed and applied immediately to column No. 2 with application of pressure by means of a squeeze bulb to force the fluid through the column as quickly as possible. The first 200 ml. of effluent are discarded and then effluent is collected in a graduated cylinder containing 1000 ml. of distilled water at room temperature. When the fluid level approaches the top of the column add sufficient 0.03 *M* NaCl so that a total of 1000 ml. of effluent can be collected.

The contents of the graduated cylinder (2000 ml., comprising 1000 ml. of effluent and 1000 ml. of distilled water) are thoroughly mixed and applied to column No. 3. After the 2000 ml. have been washed through, two column lengths (ca. 100 ml.) of 0.015 *M* NaCl are washed through, the effluent being discarded, and then about 150 ml. of 0.25 *M* NaCl are added to the column. Slightly less than one column length of fluid (ca. 35 ml.) is discarded and then collection is started. A total of 100 ml. is collected which contains most of the C′2 activity. Check ionic strength and adjust to isotonicity. Add Ca^{++} to final concentration of 0.00015 *M* and Mg^{++} to final concentration of 0.0005 *M*. Store at −20°C.

This fraction is free of detectable C′1 (tested with R1); absence of C′3a and C′3b, as well as C′4, has been demonstrated with the appropriate intermediates. Thus, the material appears to be functionally pure. However, it still contains about 10 to 15% of the total serum proteins, and most of this represents impurity. Although the product is grossly impure in the chemical sense, it serves a useful purpose as a functionally pure source of C′2.

Separation of C′3a and C′3b from Guinea Pig Serum (according to Rapp)

About 75 gm. of DEAE-cellulose are placed in a 4 liter beaker and suspended in approximately 3 liters of 0.5 *M* NaCl. The mixture is stirred well by means of a large magnetic mixing apparatus and poured into a coarse sintered glass funnel. After draining thoroughly under pressure, the cellulose is resuspended in 3 liters of 0.5 *M* NaCl and washed again. This washing process is then repeated with 0.08 *M* NaCl. The washed cellulose is resuspended in 0.08 *M* NaCl and the pH of the suspension is adjusted to 7.4 by addition of 1 *N* HCl.

About 50 gm. of CM-cellulose are washed in the same manner as DEAE-cellulose except that the pH is adjusted to 4.6 with 1 *N* NaOH.

For separation of C′3a and C′3b, three chromatographic columns are set up. Column No. 1 is a tube of 8 cm. inside diameter and 60 cm. length containing 40 gm. DEAE-cellulose in 0.08 *M* NaCl at pH 7.4. Before pouring the cellulose slurry into the tube, a plug of glass wool moistened with 0.08 *M* NaCl is inserted at the bottom. After the cellulose has settled (apply pressure with a squeeze bulb), another plug of glass wool is placed on top of the cellulose and tamped into place on top of the cellulose column. Glass beads are poured on top of the glass wool to keep it in place and to maintain the column in the properly

packed state. The column is then washed with 0.08 *M* NaCl until the effluent is free of fine particles (1 to 1½ liters usually required). During this final washing of the column, effluent is pulled through by means of suction applied to the bottom of the column. Since the column undergoes further packing during this time, it is necessary to push the glass wool plug farther down the tube in order to keep it in place on top of the cellulose. After the fluid level in the tube has reached that of the glass beads, the suction apparatus is removed and the bottom of the tube is sealed by means of a rubber sleeve stopper.

Column No. 2 is prepared in the same manner as No. 1 with 40 gm. of CM-cellulose. Instead of packing under suction, this column is prepared under pressure applied by means of a squeeze bulb. Column No. 3 contains 20 gm. DEAE in a 5 cm. inside diameter tube and is prepared under positive pressure. All operations are performed at room temperature.

Forty ml. of pooled, unabsorbed guinea pig serum are thawed just before use and mixed with 35.0 ml. of distilled water. The sleeve stopper is removed from the bottom of No. 1 tube and replaced by a rubber tube connected through a trap to the source of vacuum. Suction is applied to the tube and after the fluid level in the tube reaches the top of the cellulose column the diluted serum is poured on top of the glass beads. When the serum level reaches that of the glass wool, the tube is filled with 0.08 *M* NaCl and re-filled during collection until 6 liters of 0.08 *M* NaCl are passed through the column and collected in the trap. This effluent contains C′3a and C′2; C′3b is retained on the column.

Separation of C′3a from C′2 is accomplished by passing the 6 liters of effluent from column No. 1 through the CM-cellulose in column No. 2 under positive pressure. After the fluid level in this column reaches the bottom of the glass beads, the column is rinsed with 2 liters of 0.08 *M* NaCl. The effluent from column No. 2 contains all detectable C′2; C′3a is retained on the column. When the fluid level reaches the bottom of the glass beads, 500 ml. of 0.5 *M* NaCl are added, the first 100 ml. of effluent are discarded and the next 250 ml. retained. This solution of C′3a is placed in a beaker and while mixing constantly with a magnetic stirrer its pH is adjusted carefully to 7.1 by addition of 0.02 *N* NaOH. Store in refrigerator. For use, the salt concentration is adjusted to 0.15 *M* NaCl by dilution with water.

In order to obtain C′3b, column No. 1 is rinsed with 3 liters of 0.1 *M* NaCl which is discarded, and when the fluid level reaches the bottom of the glass beads, 500 ml. of 0.5 *N* NaCl are added. The first 100 ml. of effluent are discarded and the next 250 ml. retained. After adjusting the NaCl concentration to 0.08 *M*, the solution is added to column No. 3. When the fluid level reaches the bottom of the glass beads, 2 liters of 0.08 *M* NaCl are passed through and positive pressure is maintained until the even flow of liquid becomes interrupted by air at the narrow tip of the tube. The tube is inverted and the glass beads allowed to run out. The glass wool and cellulose are extruded on to a sheet of parafilm by application of positive pressure at the narrow end of the tube. The upper 1/3 to 1/2 of the cellulose is sliced off with a spatula and transferred to a 400 ml. beaker. The cellulose is mixed throughly with 200 ml. of 0.5 *M* NaCl and poured into a 5 cm. I.D. chromatography tube containing a glass wool plug in the bottom. As much fluid as possible is forced through and collected under positive pressure. The solution is then dialyzed against distilled water at 2-4°C., until the NaCl concentration inside the bag is 0.001 *M* or less. The resulting precipitate is collected at 0-2°C. by centrifugation at 2000 g for ten minutes. The precipitate is then extracted with 20 ml. of 0.15 *M* NaCl, centrifuged again and the supernatant fluid retained. The remaining precipitate is extracted two more times with 10 ml.

each of 0.15 *M* NaCl, centrifuging each time and retaining each supernatant fluid. The three supernates are pooled yielding 40 ml. of solution containg C′3b. Store in refrigerator.

The C′3b obtained by this procedure has been consistently free of C′3a. No detectable C′2 and only insignificant traces of C′4 have been found. C′1, as checked with R1 is absent. The C′3a fraction sometimes contains a trace of C′3b, but this can be avoided by making the initial separation at 0.07 *M* NaCl. C′2 has been found to be absent, but a small amount of C′4 has been detected (about ½% of original). C′1 appears to be absent as tested with R1.

Comments

1) Washed suspensions of the cellulose derivatives are prepared freshly each day from the dry product.
2) Approximately 1 *N* sodium hydroxide is prepared each day from saturated carbonate-free sodium hydroxide by dilution with freshly distilled water.
3) Approximately 1 *N* HCl is made by dilution from concentrated reagent grade HCl.
4) NaCl solutions are prepared freshly each day from a 3.0 *M* NaCl solution by dilution with freshly distilled water. Check concentration by conductivity measurement.
5) Chromatography tubes are cleaned with dichromate-sulfuric acid.
6) Never let the columns run dry, except when specified.
7) The chromatographic separations are made at room temperature.

COMPLEMENT FIXATION

By virtue of its capacity to combine with many antigen-antibody complexes, complement is widely used as an indicator for immunological reactions. In this manner, immune reactions can be detected even when other manifestations of antigen-antibody combination such as precipitation or agglutination are absent. A distinct advantage of complement fixation lies in the fact that either soluble or insoluble antigens may be employed. The complement fixation test depends on two properties of complement: 1) the binding or fixing of complement by antigen-antibody aggregates, and 2) the lysis of sensitized erythrocytes.

The test is performed in two steps. First, the serum to be tested for antibody, a specified dose of complement, and an appropriate amount of antigen are allowed to react at a given temperature. Sensitized sheep cells are then added and the mixture is incubated at 37°C. for sixty minutes. If the serum contains antibody to the antigen used, the complement is fixed when combination of antigen with antibody occurs and is therefore no longer available to lyse the sensitized red cells. Thus, failure to obtain lysis denotes a positive reaction, while complete hemolysis indicates a negative result, meaning that the serum did not contain antibody to the antigen employed.

The reaction may also be used to detect antigen, using a serum known to contain antibody. The principle of complement fixation may be represented by the following scheme:

Antigen + complement ⟶ (no fixation) + sensitized cells ⟶ lysis
Antibody + complement ⟶ (no fixation) + sensitized cells ⟶ lysis
Antigen + antibody + complement ⟶ (fixation) + sensitized cells ⟶ no lysis

In this manner an antibody-antigen reaction is detected by the fixation of complement, which is made visible by the erythrocyte-hemolysin indicator system.

It is essential in C′ fixation tests to employ a limited and carefully regulated quantity of C′. This requirement applies even to qualitative C′ fixation tests. The dose of C′ is specified in terms of hemolytic activity units, and hence, the factors influencing C′ activity, such as number of red cells, quantity and quality of antibody used for sensitization, ionic strength of the reaction system, concentrations of Ca^{++} and Mg^{++}, reaction time, pH and temperature, must be carefully controlled.

In the past, most C′ fixation procedures have been based on the use of two "100% units" of complement, but since 100% hemolysis does not furnish a sharply defined endpoint for the hemolytic titration of C′, in recent years numerous investigators have adopted standardization of C′ in terms of the "50% hemolytic unit," $C'H_{50}$. Experience has shown that 5 $C'H_{50}$ furnish a dose of complement which is suitable and convenient for most C′ fixation tests, whether of routine diagnostic nature, or for research purposes. If the "unknown" immune system fixes all 5 units, there will be no lysis of the sensitized erythrocytes in the second step of the test. If the "unknown" system fixes 4 units, the remaining 1 $C'H_{50}$ will produce 50% lysis of the sensitized red cells, and experience has shown that this is a useful endpoint for evaluating the result of a C′ fixation test, i.e., fixation of less than 4 out of the 5 units (as indicated by more than 50% lysis of the red cell detection system) is usually recorded as a negative reaction.

The choice of this C′ fixation endpoint is based on the desire to eliminate, or to reduce to a minimum, false positive reactions which may arise from a variety of technical factors. It is true that the sensitivity of the C′ fixation test can be increased by adoption of an endpoint involving fixation of 3 out of 5 $C'H_{50}$, or by the use of 4 $C'H_{50}$ with the endpoint based on fixation of 2 of these 4 units, but such increase in sensitivity would be achieved at the expense of reliability. In the usual diagnostic procedures, the tests are set up with relatively small volumes of reagents, delivered from serological pipettes, and pipetting errors of 10% or even 20% are not uncommon. Furthermore, some of the reagents may be slightly "anticomplementary," i.e., they may depress the hemolytic action of C′, and in addition, some deterioration of hemolytic C′ activity (10-25%, depending on experimental conditions), is likely. For these reasons, it is necessary to maintain a safety margin if technical false positive reactions are to be avoided.

The C′ fixation procedure described in the experimental part of this section has been designed for routine diagnostic purposes with these considerations in mind. It is applicable to a variety of antigen-antibody systems, and usually there is no reason why diagnostic C′ fixation tests for different infectious diseases cannot be performed by the same basic procedure, except for choice of the appropriate antigen. In situations requiring a high degree of sensitivity and/or precise quantitation, a special kind of C′ fixation analysis is called for in which the number of $C'H_{50}$ fixed by an antigen-antibody system is determined accurately by the spectrophotometric method for titration of the hemolytic activity of C′. Such a technic will also be described in the experimental part.

Since C′ can be fixed or rendered inactive by a variety of agents other than immune aggregates, a complement fixation test must be accompanied by certain controls which serve to demonstrate the specific immunological character of the fixation. Sometimes the patient's serum may inactivate C′ in the absence of antigen, and conversely, certain antigens may be encountered which interfere with the hemolytic action of C′. When sera or antigens inactivate complement alone (i.e., nonspecifically) they are said to be "anticomplementary." Consequently, it must be shown in every C′ fixation reaction that neither the serum nor the antigen alone, in

the dose employed, affects the hemolytic activity of C′.

It is well known that certain bacteria and yeasts interfere with the hemolytic action of C′, and, therefore, gross contamination of serum, antigen or the buffered saline can lead to inhibitory effects. This kind of anticomplementary action may be due to formation of immune complexes between the microorganisms and traces of corresponding antibody which may be present in the serum serving as a source of C′. Another source of anticomplementary action is aggregated gamma globulin. It was found by Davis *et al.* (165) that gamma globulin is anticomplementary, and subsequently it was shown by Olhagen (166) that aggregation of gamma globulin molecules is responsible for this effect. Recently, it has been demonstrated by Ishizaka (47) that heat-aggregated gamma globulin fixes C′ in a manner which is indistinguishable from that of antigen-antibody complexes. Sera from patients with myeloma display potent anticomplementary action (167, 168). During prolonged storage serum frequently becomes anticomplementary.

Compounds which bind Ca^{++} or Mg^{++} exert anticomplementary action if the hemolytic reaction system does not contain an adequate excess of the divalent cations. This kind of anticomplementary action no longer poses a serious problem, since the use of diluent containing ample Ca^{++} and Mg^{++} has been adopted by most investigators.

It is also known that the use of a relatively high concentration of patient's serum may sometimes exert pro-complementary action, i.e., enhance the hemolytic action of C′. If the native C′ of the patient's serum has been inactivated by adequate heating (at least 30 minutes at 56°C. ± 0.5°C.) such pro-complementary action, if encountered, is probably not due to contribution of C′ components from the patient's serum. It could arise from presence of sensitizing antibody which may enhance the lytic susceptibility of the sheep red cells in the detection system over and above that achieved by "optimal" sensitization. For this reason it is recommended that patient's serum, prior to testing, be treated with a suspension of washed sheep erythrocytes in order to remove such sensitizing antibody, if present.

These considerations place an upper limit on the amounts of serum and antigen which can be used in a C′ fixation test. With respect to serum this limit lies near 0.05 to 0.1 ml. of total quantity undiluted patient's serum in the test, for the complement fixation system described for routine diagnostic purposes in the experimental part. Somewhat more antiserum can be tolerated in Eagle's method (169).

The existence of this upper limit with respect to the quantity of patient's serum must be taken into consideration in comparative evaluation of sensitivity. While it is true that a C′ fixation test is more sensitive than a precipitin test in the sense that a positive reaction by C′ fixation requires only about 0.05 to 0.1 μg of antibody N, whereas the minimal quantity of antibody needed for a positive precipitin test is about 1 μg N, the fact that there is no upper limit in the precipitin reaction with respect to the quantity of serum, i.e., several ml. of undiluted serum may be employed, if desired, means that a lower concentration of antibody can be detected by specific precipitation than by C′ fixation.

Some investigators have used procedures of C′ fixation in which the hemolytic activity of the C′ is determined in the presence of antiserum alone, or alternatively, in the presence of antigen alone. For example, when a number of different antigens are to be compared, any anticomplementary action of the antiserum may be compensated by this device, or, in the alternate case, this may be done in tests involving comparison of several antisera reacting with a given preparation of an antigen. While there are situations where the investigator may be forced to resort to com-

pensating devices of this kind, it is usually better to avoid this. If an antigen preparation is anticomplementary it is frequently possible to remove the offending agent by purification of the antigen. For example, this situation has been encountered in the use of infected tissue culture fluid from monkey kidney cell cultures as a source of poliovirus antigen. In its native form, the infected tissue culture fluid is sometimes anticomplementary, but it is comparatively easy to isolate poliovirus from such fluids and the resulting product, even if purified only partially, gives no anticomplementary difficulty in C′ fixation tests (170).

In summary, the following rule, admittedly arbitrary, may be set down as a practical guide for avoiding, or at least minimizing, technical false positive reactions due to anticomplementary action: A positive complement fixation test observed in a mixture of antigen and antiserum is considered valid only if antigen and antiserum tested separately do not display anticomplementary action when used in *twice* the concentration as in the test proper. Another useful control device is the inclusion of antigen and antiserum control tests with 3 $C'H_{50}$, instead of the usual 5 $C'H_{50}$. This smaller dose of C′ is just sufficient for complete hemolysis and its use in the antigen and antiserum controls will reveal even slight anticomplementary activity.

The results of a typical C′ fixation test are illustrated in Table 11 for the reaction of purified type 2 poliovirus with a human convalescent serum. Two features are noteworthy. First, there is "constant antigen-cut-off," i.e., the highest dilution of antigen giving significant positive reactions (1/1280) is independent of the dilution of patient's serum. Second, the highest dilution of serum giving a positive C′ fixation reaction varies in direct proportion to the antigen dilution. This pattern, which is analogous to that seen in precipitin tests (see Chapter 2, Table 19), arises from the fact that C′ fixation, like specific precipitation, is optimal at or near the equivalence zone. The use of quantities of antigen exceeding those corresponding to equivalence zone proportions leads to inhibition, i.e., less C′ fixation. This will be discussed further in the section dealing with quantitative C′ fixation analysis.

Another example is shown in Table 12 for the reaction between bovine serum

TABLE 11

Two-dimensional Complement Fixation Test with Purified MEF-1 Virus (Preparation II-A) and Human Serum No. 300

Virus dilution	Serum dilution								Control with:	
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	5 $C'H_{50}$	3 $C'H_{50}$
1/20	0	0	0	½	4	4	4		4	4
1/40	0	0	0	0	2	4	4	4	4	4
1/80	0	0	0	0	0	3½	4	4	4	4
1/160	0	0	0	0	0	0	4	4	4	4
1/320	0	0	0	0	0	0	2	4	4	4
1/640	0	0	0	0	0	0	1	4	4	4
1/1280	½	½	½	½	½	½	3	4	4	4
1/3200	4	3	3	3	3	3	4	4	4	4
1/6400	4	4	4	4	4	4	4	4	4	4
Control with:										
5 $C'H_{50}$	4	4	4	4	4	4	4	4		
3 $C'H_{50}$	4	4	4	4	4	4	4	4		

5 $C'H_{50}$: fixation for 20 hours at 2-5°C. 0 means no lysis, 4 means complete lysis. From (170).

albumin and the corresponding rabbit antibody. In this case the quantities of antigen and antibody are given in absolute terms and it can be seen that the least quantity of antibody giving a positive reaction, with the optimal quantity of antigen, is about 0.06 μg of nitrogen (total quantity in the test system). The smallest quantity of antigen giving a positive reaction, irrespective of the quantity of antibody, is about 0.001 to 0.002 μg of nitrogen.

With antisera made by relatively short immunization, somewhat different results have been obtained, as shown in Table 13

TABLE 12

Two-Dimensional C′ Fixation Test with Bovine Serum Albumin and Homologous Rabbit Antibody (Second Course Antiserum)

Bovine serum albumin N per test mixture, μg	Antibody Nitrogen per Test Mixture, μg									
	4	2	1	0.5	0.25	0.125	0.063	0.031	0.016	0
1	0	0	1	1	4	4	4	4	4	4
0.5	0	0	0	0	4	4	4	4	4	4
0.25	0	0	0	0	4	4	4	4	4	4
0.125	0	0	0	0	1	4	4	4	4	4
0.063	0	0	0	0	0	3	4	4	4	4
0.031	0	0	0	0	0	1	4	4	4	4
0.016	0	0	0	0	0	0	4	4	4	4
0.008	0	0	0	0	0	0	2	4	4	4
0.004	0	0	0	0	0	0	1	4	4	4
0.002	0	0	0	0	0	0	3	3½	4	4
0.001	3	3	3	3	3	3	3	3½	4	4
0	4	4	4	4	4	4	4	4	4	4

5 $C'H_{50}$; total fixation volume = 1.3 ml.; 0.2 ml. of sensitized red cells standardized as described in experimental part; fixation for 20 hours at 2 to 4° C.; hemolytic reaction 60 min. at 37° C.; readings given as degree of hemolysis, with "0" representing no lysis, "4" representing complete lysis. From (126).

TABLE 13

Two-Dimensional C′ Fixation Test with Bovine Serum Albumin and Homologous Rabbit Antibody (First Course Antiserum)

Bovine serum albumin N per test mixture, μg	Antibody N per Test Mixture, μg									
	4	2	1	0.5	0.25	0.125	0.063	0.031	0.016	0
1	0	0	0	3	4	4	4	4	4	4
0.5	0	0	0	0	4	4	4	4	4	4
0.25	0	0	0	0	4	4	4	4	4	4
0.125	0	0	0	0	4	4	4	4	4	4
0.063	0	0	0	0	4	4	4	4	4	4
0.031	0	0	0	½	4	4	4	4	4	4
0.016	0	0	0	½	4	4	4	4	4	4
0.008	0	0	0	1	4	4	4	4	4	4
0.004	0	0	4	4	4	4	4	4	4	4
0.002	2	2	4	4	4	4	4	4	4	4
0	4	4	4	4	4	4	4	4	4	4

5 $C'H_{50}$; total fixation volume = 1.3 ml.; 0.2 ml. of sensitized red cells standardized as described in experimental part; fixation for 20 hours at 2 to 4°C.; hemolytic reaction 60 min. at 37°C.; readings given as degree of hemolysis, with "0" representing no lysis, "4" representing complete lysis From (126).

for the system bovine serum albumin-rabbit antibody. In the case of this antiserum the least quantity of antibody giving a positive reaction was 0.5 μg N, and the optimally reactive level of antigen shifted to a higher concentration, approximately 0.1 μg N.

The C′ fixation pattern for the reaction between pneumococcus type 3 capsular polysaccharide and homologous rabbit antibody is illustrated in Table 14.

It is evident from these examples that the C′ fixation titer of antiserum depends on the quantity of antigen used in the test, and therefore, for any given serological system it is necessary to determine the level or range of antigen concentration yielding the highest antiserum titer. In crude antigen preparations, for example, virus-infected tissue culture fluids, the antigen concentration is sometimes too low to achieve the optimal level of antigen. In such cases, purification and concentration of the antigen is indicated.

In choosing the appropriate antigen concentration, the possibility of variation in the qualitative C′ fixing characteristics of the antibody should be kept in mind. This matter is discussed more fully in the section dealing with quantitative C′ fixation analysis.

For interpretation of checkerboard patterns of C′ fixation, such as those in Tables 11 to 14, it is convenient to draw a line connecting all points showing 50% lysis. These points are located approximately by interpolation between the appropriate experimental readings. The usefulness of the resulting line, known as an "iso-fixation" curve, for comparing the potencies and "avidities" of different antisera, has been pointed out by Rapport and Graf in studies with rat lymphosarcoma lipids (268).

Quantitative C′ Fixation Analysis (122–128)

The method of C′ fixation discussed thus far is based on titration in terms of dilution endpoint. The development of a precise spectrophotometric method for the hemolytic measurement of C′ made it possible to design a C′ fixation analysis which furnishes results at a level of precision approaching that of the quantitative precipitin method. This C′ fixation analysis is not intended for use in routine diagnostic tests, but represents, primarily, a research tool. In essence, the procedure involves the use of a relatively large quantity of C′ (50, 100 or even 200 $C'H_{50}$) and quantities of immune reagents chosen so that only part of the C′ is fixed, leaving free C′ which can be determined accurately by photometric analysis of hemolytic activity.

TABLE 14

Titration of C′ Fixing Activity by the Dilution Method (Rabbit Anti-Pneumococcal Serum Type III Reacting with SIII)

		Antibody Nitrogen Per Test Mixture, μg							
		4	2	1	0.5	0.25	0.13	0.06	0
SIII per test mixture, μg	0.4	0	0	1	4	4	4	4	4
	0.2	0	0	0	2	4	4	4	4
	0.1	0	0	0	0	4	4	4	4
	0.05	0	0	0	0	2	4	4	4
	0.025	0	0	0	0	0	4	4	4
	0.013	0	0	0	0	0	3	4	4
	0.006	0	0	0	0	0	2	4	4
	0.003	0	0	0	0	0	1	4	4
	0.0015	3	3	3	3	3½	3½	4	4
	0	4	4	4	4	4	4	4	

(Fixation for 30 min. at 37°C. with 5 $C'H_{50}$)

For the sake of accuracy of pipetting, relatively large volumes of reagents are employed. Thus, in the first step of the analysis (fixation stage), 2.5 ml. of an appropriate dilution of antiserum, 5.0 ml. of a dilution of guinea pig serum furnishing 100 $C'H_{50}$, and 2.5 ml. of antigen, at a suitable concentration, are admixed and incubated for ninety minutes at 37°C., or kept for twenty hours at 2-4°C. At least three controls are required: 1) 100 $C'H_{50}$ mixed with diluent, 2) 100 $C'H_{50}$ mixed with antigen, and 3) 100 $C'H_{50}$ mixed with antiserum. At the end of the fixation period, portions of each of the reaction mixtures are diluted accurately with ice-cold diluent and the number of residual $C'H_{50}$ is determined with suitable aliquots by the photometric method for measuring the hemolytic activity of C'. The values of C' activity in the controls are averaged, unless there are serious discrepancies, in which case the analysis is invalid, and the number of residual $C'H_{50}$ found in the reaction mixtures is subtracted from the average control value, yielding the number of $C'H_{50}$ fixed.

A typical protocol is shown in Table 15. Evaluation of the residual $C'H_{50}$ from the degree of hemolysis can be made by application of the conversion factors (Table 3), but graphical evaluation is preferable since it does not entail the assumption that $1/n = 0.2$. However, this is not a serious

TABLE 15

Fixation of C' by a Constant Amount, 2.83 μg Antibody N, of Rabbit Antipneumococcus Type III Serum C-28 and Varying Amounts of S III. C' Added, 50 C' H_{50}; Fixation, 60 Min. at 37°C.; lysis, 60 Min. at 37°C.

Controls	Reaction mixture used for hemolytic estimation with 1.0 ml. sensitized cells		Veronal buffer added to make 7.5 ml. total volume	Hemolysis	Factor for calculating C' H_{50}	C' H_{50} Left in entire reaction mixture	C' H_{50} Fixed*
	Diln.	Vol.					
		ml	*ml*	%			
C' and veronal buffer	1→10	2.0	4.5	51.0	1.01	50.5	
		2.5	4.0	72.0	1.205	48.2	
C' and S III	1→10	2.0	4.5	51.5	1.013	50.7	
		2.5	4.0	71.4	1.20	48.0	
C' and antiserum	1→10	2.0	4.5	51.0	1.01	50.5	
		2.5	4.0	70.2	1.185	47.4	
Fixation of C' by 2.83 μg antibody N and					mean = 49.2		
0.15 μg S III	1→5	2.0	4.5	32.7	0.861	21.5	29
		3.0	3.5	72.8	1.215	20.3	
0.4 μg S III	1→5	3.0	3.5	49.6	0.997	16.6	33, 34†
		4.0	2.5	80.0	1.32	16.5	
0.5 μg S III	1→5	2.0	4.5	27.4	0.82*	20.5	30, 31, 32
		3.0	3.5	67.5	1.155	19.3	
0.6 μg S III	1→10	4.0	2.5	39.8	0.920	23.0	27, 28
		5.0	1.5	64.8	1.128	22.6	
1.0 μg S III	1→10	3.0	3.5	45.1	0.962	32.1	18
		4.0	2.5	74.5	1.235	30.9	

*All values are corrected to 50 $C'H_{50}$ (i.e., × 50 ÷ 49.2) and rounded off to the nearest whole number.
†More than one value is given to indicate reproducibility in similar runs on different days. From (122).

TABLE 16

Effect of Variations in the Amount of C′ and Immune System on 1/n Rabbit Antipneumococcus Type III Serum C-28 Plus S III Fixation, 90 Min. at 37°C.; Lysis, 60 Min. at 37°C.

Antibody N	$C'H_{50}$ in control tubes	S III	Reaction mixture used for hemolytic estimation		Lysis	1/n	$C'H_{50}$ Fixed
			Diln.	Vol.			
μg 1.9	60	*μg* none	1→24	*ml.* 3.0	*per cent* 21.8		
				3.5	31.9		
				4.0	49.1		
				4.5	65.3		
				5.0	75.6	0.204	none
0.95	26	none	1→10	3.0	21.8		
				3.5	37.8		
				4.0	53.8		
				4.5	67.9		
				5.0	77.8	0.203	none
0.48	12	none	1→6	3.0	9.9		
				3.5	17.0		
				4.0	27.3		
				4.5	39.8		
				5.0	51.3	0.194	none
1.9	60	0.2	1→12	3.5	36.2		
				4.0	51.5		
				4.5	64.9		
				5.0	77.0		
				5.5	82.5	0.208	30
0.95	26	0.1	1→5	3.0	36.0		
				3.5	56.0		
				4.0	68.9		
				4.5	79.4		
				5.0	84.7	0.215	11
0.48	12	0.05	1→3	3.0	28.7		
				3.5	46.3		
				4.0	63.7		
				4.5	77.0		
				5.0	84.9	0.190	4

From (122).

source of difficulty, since it has been found, as shown in Table 16 that the value of 1/n does not vary significantly from 0.2, even when most of the available C′ has been fixed.

Fixation of C′ as a Function of Antigen Concentration, with Antibody Kept Constant: A plot of the extent of C′ fixation as a function of antigen, with antibody kept constant, bears a striking resemblance to that for specific precipitation. In both instances, peak values are attained with a slight excess of antigen (as judged in terms of the precipitin system), and inhibition of C′ fixation, or of precipitation, occurs as the quantity of antigen is increased substantially beyond that corresponding to equivalence zone proportions. A typical family of C′ fixation curves is shown in Figure 68 for the reaction between pneumococcus SIII and anti-pneumococcus Type III rabbit serum (123). A similar set of curves is shown in Figure 69 for the system hen egg albumin

FIG. (
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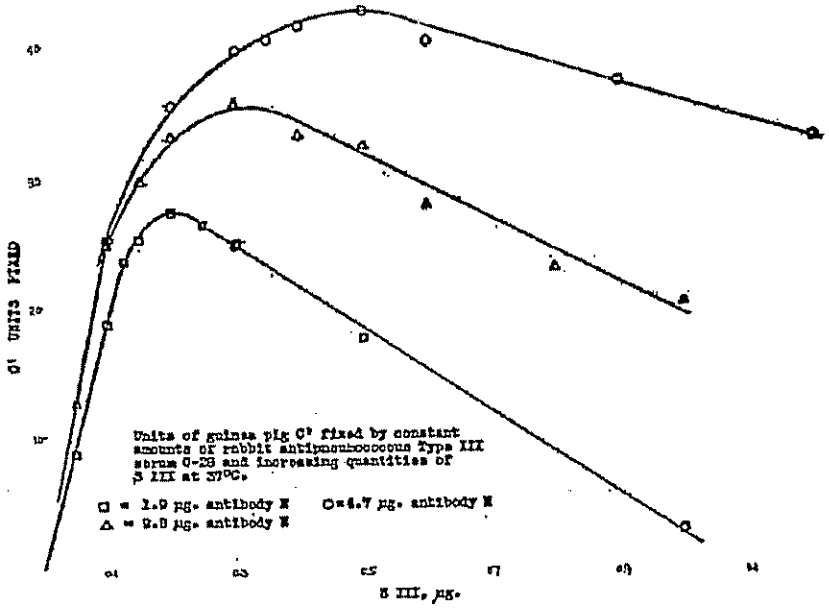

FIG. 68. Fixation of guinea pig complement by constant amounts of rabbit antipneumococcus Type 3 serum and varying quantities of pneumococcal capsular polysaccharide, S III, at 37° C. From (123).

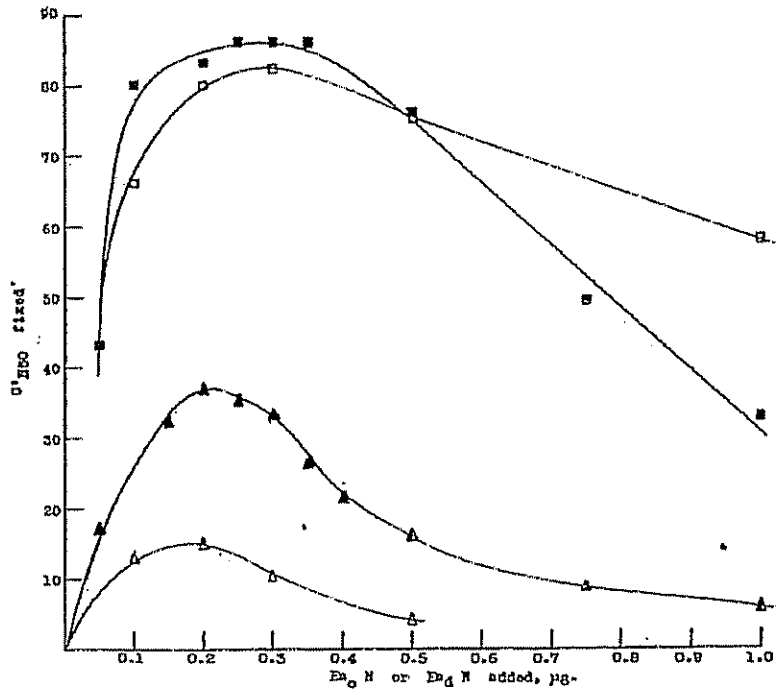

FIG. 69. Fixation of C′ by varying quantities of Ea$_o$ and 2.0 μg of antibody N from rabbit anti-Ea$_o$ serum 791 at 37°C. (▲) and at ice box temperatures (■). Fixation of C′ by varying quantities of Ea$_d$ and 2.0 μg of cross reacting antibody N from serum 791 at 37°C. (Δ) and in the cold (□). From (125).